

A phylogeny of *Cribrariaceae* among *Myxomycetes* derived from morphological characters

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Abstract – A cladistic study of the *Cribrariaceae* was performed to examine its phylogenetic position among the *Myxomycetes* based on a data matrix comprising 54 morphological characters and 55 exemplar myxomycete species. Parsimony ratchet with implied weighting was employed as tree search strategy. Results show the *Cribrariaceae* as a monophyletic group that includes *Cribraria* and *Dictydium* but not *Lindbladia* and suggest *Trichiales* as the sister group. Our analyses neither support *Dictydium* as a genus separated from *Cribraria* nor the *Liceales* as monophyletic. This is the first attempt to evaluate the phylogenetic relationships of this group using morphological characters from representative species of all *Myxomycetes* within a cladistic framework.

Keywords – *Echinosteliales*, *Physarales*, slime molds, *Stemonitales*, taxonomy

Introduction

According to Alexopoulos (1973), Zopf (in *Die Pilzthiere oder Schleimpilze*, 1885) included *Cribrariaceae* within the *Endosporeae* in the division *Eumycetozoa* based on the presence of swarm cells, true plasmodia, and the formation of spores in sporocysts. Masee (1892), who based his classification on the capillitium as the primary taxonomic criterion, divided the *Myxogastres* into four orders, placing *Cribrariaceae* in order *Peritricheae* suborder *Cribrariae* together with the suborder *Tubulinae*. In contrast, Lister (1894), using spore color as primary criterion and presence/absence of lime and capillitial structure as secondary criteria, included the *Cribrariaceae* within Sub-class II *Endosporeae* in the Cohort II-*Lamprosporales* (spores not violet brown) and Sub-cohort I *Anemineae* (no capillitium). Macbride & Martin (1934) divided the *Myxomycetes* into four orders: *Physarales*, *Stemonitales*,

Trichiales and *Liceales*, placing the *Cribrariaceae* in the *Liceales*. Most modern authors (Martin et al. 1983, Nannenga-Bremekamp 1991, Stephenson & Stempen 1994, Lado & Pando 1997, Keller & Braun 1999) include six orders (*Ceratiomyxales*, *Echinosteliales*, *Physarales*, *Stemonitales*, *Trichiales*, *Liceales*) in the *Myxomycetes*, although *Ceratiomyxales* are currently recognized as most closely related to the protostelids (Olive 1975, Spiegel 1990, Spiegel et al. 1995). *Cribrariaceae* is classified together with *Liceaceae* and *Reticulariaceae* in Subclass *Myxogastromycetidae* in *Liceales* (Martin 1960, Martin & Alexopoulos 1969).

The order *Liceales* is traditionally characterized mainly by the absence of a true capillitium (Eliasson 1977). The *Cribrariaceae* was established in 1838 (Hawksworth et al. 1995) to accommodate species possessing: *i*) a netlike covering that extends over either the entire surface of the fruiting body spore mass or upper sporotheca and *ii*) the presence of minute conspicuous granules. Although we refer to the latter as “plasmodic granules” (Lister 1911, 1925) based on their plasmodic origin, these are also known as “dictydine granules” (Martin 1949, Martin & Alexopoulos 1969) or “lime globules” (Nannenga-Bremekamp 1991). Schoknecht (1975) revealed the presence of calcium in the plasmodic granules, but their chemical structure is still unknown. At present, the netlike covering and plasmodic granules are diagnostic for the *Cribrariaceae*, even though *Lindbladia tubulina*, *Cribraria zonatispora* (Lado et al. 1999), and *C. fragilis* (Estrada-Torres et al. 2001) lack a peridial net. All species in this family (with a few exceptions, such as *Lindbladia tubulina*) also produce stalked sporangia as fruiting bodies.

The *Cribrariaceae* can include either two (*Cribraria*, *Lindbladia*) or three (*Cribraria*, *Dictydium*, *Lindbladia*) genera. For example, North American researchers following Lister (1925) recognize *Dictydium* as a separate genus (Martin 1949, Martin & Alexopoulos 1969, Farr 1976, Stephenson & Stempen 1994, Keller & Braun 1999), while most Europeans include *Dictydium* within *Cribraria* (Eliasson 1977, Nannenga-Bremekamp 1991, Lado & Pando 1997). Both taxonomic schools include *Lindbladia* in the *Cribrariaceae*. *Lindbladia tubulina*, which has been referred to other genera — *Aethalium*, *Enteridium*, *Licea*, *Perichaena*, *Physarum*, *Tubulina* (Martin & Alexopoulos 1969) — is a variable taxon that forms true aethalia at one extreme but intergrades into pseudoaethalia; sporangia vary from closely spaced to (usually) sessile or (rarely) short-stalked (Hatano et al. 1996). Although *Lindbladia tubulina* has no peridial network, the presence of plasmodic granules maintains its placement in the *Cribrariaceae* (Martin & Alexopoulos 1969). Until now, there has been no phylogenetic study to evaluate either the monophyly of the *Liceales* or the relationships among genera of *Cribrariaceae* and with other groups of *Myxomycetes*. Schoknecht (1975) suggested that the presence of calcium in

the plasmodic granules might indicate a possible relationship between the *Cribrariaceae* and the *Physaraceae*, but there has been no study to demonstrate this relationship in a phylogenetic context. The main goal in this paper is to use cladistics analysis of morphological characters to evaluate which genera belong to the *Cribrariaceae* and to explore the phylogenetic position of *Cribrariaceae* among *Myxomycetes*.

Materials and methods

Choice of terminal units

Representative species of all *Myxomycetes* were included in this study. We selected a total of 55 exemplar species representing each order and family as classified in Martin et al. (1983) for which complete specimens permitted observation of all characters. Terminal units comprised 5 *Trichiales*, 6 *Stemonitales*, 8 *Physarales*, and 4 *Echinosteliales*. Of the *Liceales*, 17 species represented *Cribrariaceae*, 7 *Liceaceae*, and 6 *Reticulariaceae*. We also selected as outgroups two species of *Ceratiomyxales*, an order currently phylogenetically placed in the protostelids (Olive 1975, Spiegel 1990, Spiegel et al. 1995). Taxonomic names follow Hernández-Crespo & Lado (2005). All specimens studied belong to the herbarium TLXM at the Universidad Autónoma de Tlaxcala (see TABLE 1).

TABLE 1. List of specimens examined

ORDER	TERMINAL UNITS AND REPRESENTATIVE SPECIMENS
<i>Ceratiomyxales</i>	<i>Ceratiomyxa fruticulosa</i> (O.F. Müll.) T. Macbr. ET-4676, ET-2536, RP-2672, S. L. Stephenson-7405 <i>Ceratiomyxa morchella</i> A.L. Welden ET-3419, ET-3602
<i>Trichiales</i>	<i>Calomyxa metallica</i> (Berk.) Nieuwl. RP-1566, GF-1134 <i>Calonema foliicola</i> Estrada et al. ET-8159, ET-8286, ET-4532 <i>Hemitrichia calyculata</i> (Speg.) M.L. Farr RP -2582, ET-4081b, ET-4220 <i>Metatrichia vesparia</i> (Batsch) Nann.-Bremek. RP-2361, ET-4520 <i>Trichia decipiens</i> (Pers.) T. Macbr. HC-718, MAFungi-27069, S. L. Stephenson-7406
<i>Stemonitales</i>	<i>Comatricha laxa</i> Rostaf. VG-580, HC-1384, HC-1582, RP-1559 <i>Enerthenema papillatum</i> (Pers.) Rostaf. RP-35, GF-203, MAFungi-17206 <i>Lamproderma scintillans</i> (Berk & Broome.) Morgan ET-4612a, RP-1076, RP-251 <i>Stemonaria longa</i> (Peck) Nann.-Bremek. et al. RP-2149, ET-6037 <i>Stemonitis pallida</i> Wingate ET-10308, ET-4470 <i>Stemonitopsis typhina</i> (F.H. Wigg.) Nann.-Bremek. RP-2197a, ET-4250, ET-4901, ET-4401
<i>Physarales</i>	<i>Diachea leucopodia</i> (Bull.) Rostaf. ET-4734, RP-2603, ET-4393 <i>Didymium serpula</i> Fr. RP-1782 <i>Elaeomyxa cerifera</i> (G. Lister) Hagelst. HC-1706, GF-826 <i>Lepidoderma tigrinum</i> (Schr.) Rostaf. HC-1521, HC-1700, HC-2302 <i>Physarella oblonga</i> (Berk. & M.A. Curtis) Morgan ET-5030, RP-2525, ET-4615, ET-4960 <i>Physarum bogoriense</i> Racib. ET-4617, ET-10514, ET-4301a <i>Physarum flavicomum</i> Berk. ET-6080 <i>Willkommlangea reticulata</i> (Alb. & Schwein.) Kuntze ET-3590
<i>Liceales</i>	<i>Cribraria argillacea</i> (Pers. ex J.F. Gmel.) Pers. GF-535, GF-365, RP-1999 <i>Cribraria atrofusca</i> G.W. Martin & Lovejoy HC-870, GF-728, GF-940 <i>Cribraria aurantiaca</i> Schrad. TNS-2612 <i>Cribraria cancellata</i> (Batsch) Nann.-Bremek. RP-2199a <i>Cribraria fragilis</i> Lado & Estrada E. Conde-pw17

TABLE 1, CONCLUDED

	<i>Cribraria microcarpa</i> (Schr.) Pers. TNS-3445
	<i>Cribraria mirabilis</i> (Rostaf.) Massee GF-44, MAFungi-17473
	<i>Cribraria oregana</i> H.C. Gilbert RP-1868, RP-1100, HC-183
	<i>Cribraria piriformis</i> Schrad. HC-2285, RP-1523, RP-227, MAFungi-27001
	<i>Cribraria purpurea</i> Schrad. HC-1589, HC-815, HC-171, HC-497, HC-892, HC-1869
	<i>Cribraria rufa</i> (Roth) Rostaf. MAFungi-27121
	<i>Cribraria splendens</i> (Schr.) Pers. GF-1769, GF-396, HC-110
	<i>Cribraria tenella</i> Schrad. ET-4448, ET-4307, ET-4229
	<i>Cribraria violacea</i> Rex ET-4740b, ET-4863, RP-2147
	<i>Cribraria vulgaris</i> Schrad. GF-1356, HC-2031, HC-2075, GF-1772
	<i>Cribraria zonatispora</i> Lado et al. E. Conde-pm56
	<i>Dictydiaethalium plumbeum</i> (Schumach.) Rostaf. ET-3562
	<i>Licea biforis</i> Morgan EC- p55, S. L. Stephenson-7422
	<i>Licea castanea</i> G. Lister VG-346, VG-561, VG-471, VG-726
	<i>Licea minima</i> Fr. Vázquez-García 2, MAFungi-27131
	<i>Licea parasitica</i> (Zukal) G.W. Martin HC-2006, VG-444, VG-462
	<i>Licea pusilla</i> Schrad. RP-1058, RP-179, GF-417
	<i>Licea pygmaea</i> (Meyl.) Ing RP-2017, HC-232, HC-783, VG-343
	<i>Licea variabilis</i> Schrad. RP-2300
	<i>Lindbladia tubulina</i> Fr. GF-769, RP-195
	<i>Lycogala conicum</i> Pers. Autun-71
	<i>Lycogala epidendrum</i> (L.) Fr. RP-1458, GF-218, MAFungi-20556, S. L. Stephenson-7412
	<i>Reticularia olivacea</i> (Ehrenb.) Fr. RP-978, MAFungi-17202
	<i>Reticularia splendens</i> Morgan RP-743, GF-896
	<i>Tubulifera arachnoidea</i> Jacq. RP-542, GF-148, MAFungi-17468, 17401, S. L. Stephenson-7416
<i>Echinosteliales</i>	<i>Clastoderma debaryanum</i> A. Blytt ET-6328
	<i>Clastoderma pachypus</i> Nann.-Bremek. ET-5378
	<i>Echinostelium arboreum</i> H.W. Keller & T.E. Brooks GF-256
	<i>Echinostelium minutum</i> de Bary ET-892

Morphological data matrix

Characters were selected and analyzed based on the variation observed among species and previous reports in the literature, without excluding a priori any source of information. Morphological observations were analyzed and interpreted in the framework of cladistic epistemology (De Pinna 1991, De Luna & Mishler 1996). Hypotheses of homology were based on similarity, conjunction, independence, variability, and heritability as the principal criteria proposed by De Pinna (1991). Empirical delimitation of characters and states should be considered our best estimates and potentially subject to modification and rejection, as it should be for all characters in any cladistic analysis.

Variable morphological characters were scored into at least two states (see APPENDIX 2). Characters often used to classify *Myxomycetes* (Martin & Alexopoulos 1969) included sporotheca features (e.g., sporocarp morphology, capillitial type, sporophore arrangement, spore ornamentation, type of lime in the peridium, presence of plasmodic granules, surface net, color of spores and capillitium). Potentially useful characters still unknown for most species (e.g., plasmodium, swarm cells ultrastructure) were excluded. Of the selected morphological characters, 46 were binary coded and 8 were multi-state (APPENDIX 2). The data matrix comprising 55 terminal units and 54 characters was constructed in MacClade 4.05 (Maddison & Maddison 2002) (see APPENDIX 1). Multi-

state characters were kept unordered. Characters with more than one state in a single terminal unit were coded as polymorphic.

Phylogenetic analyses

All characters were initially weighted equally (EW analyses). We used PAUPRat (Sikes & Lewis 2001) to implement a parsimony ratchet search using PAUP 4.0b10 (Swofford 2000) on a Macintosh G4 iBook. The parsimony ratchet is efficient at finding trees for data sets too large for traditional heuristic search methods (Nixon 1999). Following author recommendations, we performed several searches, creating multiple folders (=20), each with a separate batch file, with 200 iterations perturbing just 10% of the characters (Goloboff 1997). Trees from each search were collected into a single file and filtered. A strict consensus was calculated on these EW final trees.

Character states were optimized with the ACCTRAN option on a tree selected by an additional search with implied weighting in PAUP 4.0b10. Jackknife values (Farris et al. 1996) and Bremer index (Bremer 1994) measured the relative support of clades. Jackknife values were estimated with 10% of the characters deleted using the "fast" stepwise-addition option and repeated 10 times with 10000 replicates in PAUP. Bremer values were generated using Auto Decay version 4.0.2 (Eriksson 1999) over PAUP.

Implied weighting

Farris (1983) noted that because not all characters provide equal phylogenetic information, some characters deserve more weight than others. We used implied weighting (IW) to assess the effects of weighting against homoplastic characters. This weighting scheme uses evidence on homoplasy to estimate character reliability (Goloboff 1997). A character that operates as an uncontroversial synapomorphy (no reversals, no parallelism, therefore no homoplasy) will have a CI (consistency index) or RI (retention index) of 1.0, whereas a character with some homoplasy will have lower fit (Wenzel 2002). The IW was calculated in PAUP 4.0b10, holding 100 trees in each replicate. Instead of minimizing the length of a cladogram during the search of the most parsimonious tree, the value to be maximized under the implied weighting procedure is the F_{IT} or the sum of the F_{IT} of each individual character in a given tree. The F_{IT} was determined by a decreasing concave function that accounts for the homoplasy (i.e. extra steps) of a character i in the tree under evaluation, and a constant, K , that defines the concavity of the function (Goloboff 1993). The concavity of the function is steeper at lower values of K and so penalizes more strictly the homoplastic characters. At higher values of K , the function becomes asymptotically similar to the linear function of equal weights. So far, the decision concerning on how strongly to weight against homoplasy has been subjective (Lopardo 2005). We performed several analyses under different concavity values until the tree did not change further. We found the best- F_{IT} character with a $K=20$ value.

Results

Phylogenetic analyses

The EW analyses produced 875 equally most parsimonious trees after filtering, each 167 steps long and with a consistency index (CI) of 0.461 and retention index (RI) of 0.728. The EW strict consensus tree was unresolved

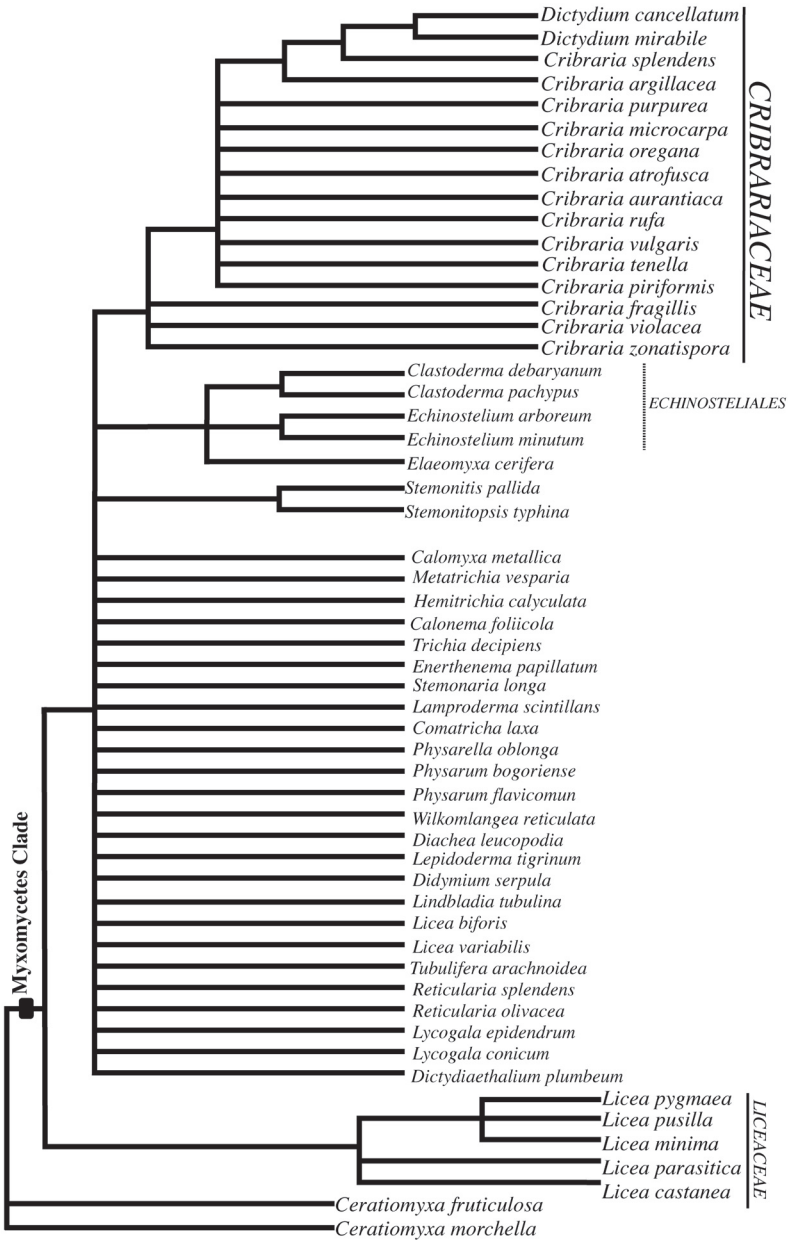


FIGURE 1. Strict unresolved consensus tree from an analysis using equal character weight.

for the deepest branches; nevertheless, four clades were recovered (FIG. 1): here referred as the *Cribrariaceae* (16 terminals without *Lindbladia*), the *Echinosteliales* (4 terminals) plus *Elaeomyxa cerifera*, the *Stemonitis* clade (2 terminals), and the *Liceaceae* clade (only 5 terminals).

The IW analysis generated seven trees with a best fit of -37.473 and a length of 167 steps, the same as the EW shortest trees. The strict consensus of IW trees (FIG. 2) is used below (see Discussion) to compare the relationships of *Cribrariaceae* and the remaining *Myxomycetes*.

The IW strict consensus revealed several taxonomically meaningful clades, including four main clades (FIG. 2). Clade 1 comprises the *Stemonitales*, *Echinosteliales*, and *Physarales* sensu Martin et al. (1983), a relationship found also by a previous molecular-based analysis (Fiore-Donno et al. 2008). Within clade 1, Sub-clade A shows the *Stemonitales* (S_A) and *Echinosteliales* (E_A) plus *Elaeomyxa cerifera* as sister groups. The phylogenetic position of *Diachea leucopodia* and *Lepidoderma tigrinum* is undefined within Subclade A. Sub-clade B comprises part of the *Physarales* sensu Martin et al. (1983). Jackknife analysis indicated a low-level support for Clade 1; only three clades (the *Stemonitis pallida* plus *Stemonitopsis typhina*, and the genera *Clastoderma* and *Echinostelium*) show Jackknife support above 80%. Most clades within Clade 1 were not supported by decay analysis either, and only the *Stemonitis pallida* plus *Stemonitopsis typhina* clade had a low Bremer support (= 2).

Clade 2 is sister to Clade 1 and encompasses three clades. The *Reticulariaceae* sensu Martin et al. (1983) plus *Lindbladia tubulina* and *Tubulifera arachnoidea* is a monophyletic group, which we call the *Reticulariaceae* (R). The Jackknife and Bremer support for this clade is low (60%, 2). The sister clade of this group is formed by *Calomyxa metallica* plus *Licea variabilis* and *Licea biforis* (part of *Liceaceae*, FIG. 2). A third clade composed of *Hemitrichia calyculata* and *Trichia decipiens* (part of *Trichiales*, FIG. 2) is sister to the *Reticulariaceae* and the *Calomyxa metallica* + *Licea variabilis* + *Licea biforis* clades.

In Clade 3, the strict consensus shows all 16 *Cribraria* species as a monophyletic group. We refer this as the *Cribrariaceae* clade (C). The Jackknife and Bremer support for this clade is 85% and 2 respectively. Sister to the *Cribrariaceae* clade is *Calonema foliicola* and (more basally) *Metatrachia vesparia* (both exemplars of the *Trichiales*).

Finally, Clade 4, here labeled as the *Liceaceae* clade (L), comprises five species of *Licea* sensu Martin et al. (1983). This is the basal group of the *Myxomycetes* clade (Jackknife value = 76%; Bremer support = 2).

Discussion

The IW results indicate monophyly for the *Stemonitales* and *Echinosteliales* but not for the *Physarales*, *Trichiales*, or *Liceales*, which appear either paraphyletic (*Physarales*) or polyphyletic (*Trichiales*, *Liceales*) (FIG. 2).

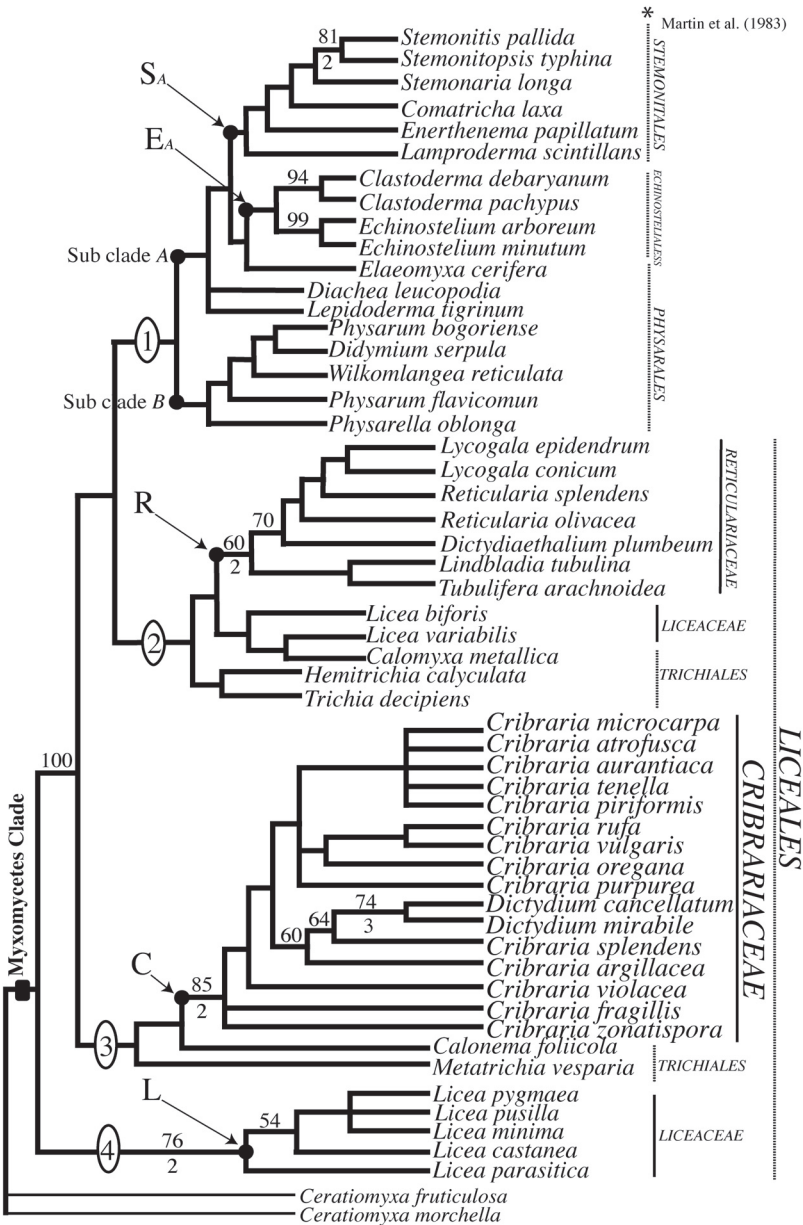


FIGURE 2. The strict consensus from seven trees using implied weights. Numbers in circles denote the principal clades considered here. Numbers above branches indicate Jackknife values; numbers below branches indicate the Bremer support.

Liceales are polyphyletic

Our results suggest that the *Liceales* sensu Martin et al. (1983) are not monophyletic. The polyphyletic origin of the *Liceales* has been proposed before (Eliasson 1977). Currently, the order is delimited mostly by the lack of capillitium. However, Alexopoulos (1976), noting that absence of a capillitium was not a good taxonomical character, suggested that this character should be re-evaluated because other myxomycete species with small sporotheca occasionally lack a capillitium [e.g., some *Perichaena* species (*Trichiales*), Keller & Eliasson 1992; *Didymium eremophilum* (*Physarales*), Blackwell & Gilbertson 1980]. In contrast, small peridial inner projections have been found in some species of *Liceaceae* that may be perceived as the remains of a rudimentary capillitium (Alexopoulos 1976, Gilert 1985, Eliasson et al. 1991). Another example is *Listerella paradoxa*, a species currently included in *Liceales*, in which capillitium is present in the fructifications (Eliasson & Gilert 1982, Martin et al. 1983).

Eliasson (1977) and Eliasson et al. (1991) suggested that the assimilative stage, either a protoplasmodium or a phaneroplasmodium, might also indicate the heterogeneity of the *Liceales*. Unfortunately, because this information is unknown for the species we studied due to difficulties presented by axenic laboratory culture, it was not included in the analyses.

The results of the present study indicate that families currently classified in the order *Liceales* — *Reticulariaceae* (in Clade 2), *Liceaceae* (in Clades 2 and 4), *Cribrariaceae* (in Clade 3) — do not share a common ancestor (FIG. 2). Our analyses also shed some light on the taxonomic status of these three families.

In the character optimization, one unique synapomorphy— presence of a pseudoaethalium (character 4:1) — supports the monophyly of the *Reticulariaceae* (FIG. 3). All genera (*Lycogala*, *Reticularia*, *Dictydiaethalium*, *Tubulifera* = *Tubifera*) included in clade *R* have been traditionally recognized as part of *Reticulariaceae*, except for *Lindbladla*, a genus that has been classified in the *Cribrariaceae* (Martin & Alexopoulos 1969, Martin et al. 1983, Nannenga-Bremekamp 1991, Stephenson & Stempen 1994).

With respect to the monotypic *Liceaceae* sensu Martin et al. (1983), the genus *Licea* appears polyphyletic with some exemplar species shown in Clade 2 and others in Clade 4 (FIG. 2). *Licea variabilis* (in Clade 2) is non-typical compared to most *Licea* species in sporophore form (Martin & Alexopoulos 1969) and development of a phaneroplasmodium (McManus 1966). The *Liceaceae* in Clade 4 (*L. pygmaea*, *L. pusilla*, *L. minima*, *L. castanea*, *L. parasitica*) have the unique synapomorphy of a myxospore wall that is thinner at one pole (character 51:1), a character not present in *Licea biforis* and *L. variabilis*. These two taxa and *Calomyxa metallica* (in Clade 2) are supported by the presence of a sporocarp (character 3:1, FIG. 3).

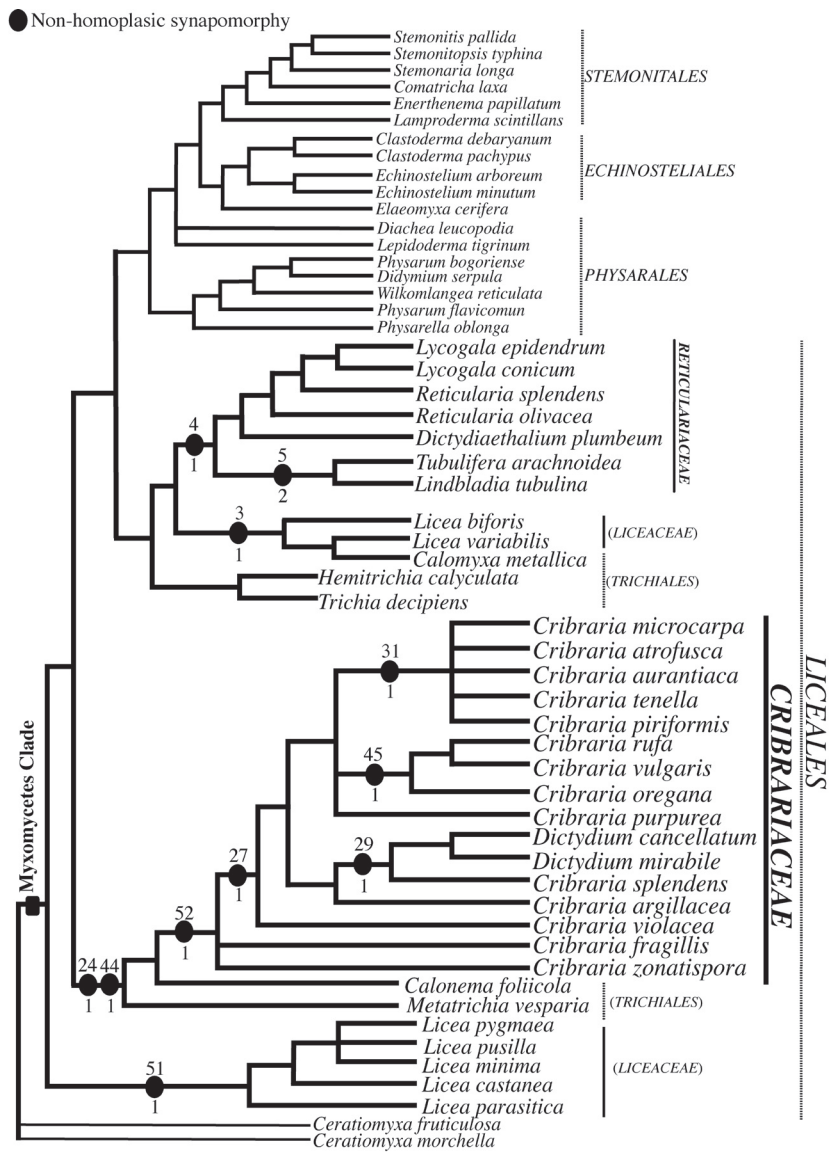


FIGURE 3. One of the most parsimonious trees encountered during implied weights search, showing the character states that can be unambiguously optimized. Numbers above dots indicate the character and number below dots indicate the character states.

Several workers (Alexopoulos 1973, Alexopoulos 1982, Fiore-Donno et al. 2005) have suggested a relationship between *Trichiales* and *Liceales* based on either the plasmodial common to both groups (Alexopoulos 1973, 1982) or molecular phylogenetic analysis (Fiore-Donno et al. 2005).

Phylogenetic position of *Cribrariaceae*

Our cladistic analyses support the monophyly of the *Cribrariaceae* (Clade C, FIG. 2). According to Martin (1949), Martin & Alexopoulos (1969), and Nannenga-Bremekamp (1991), plasmodic granules, the lack of a capillitium, and the persistent surface net in *Cribraria* species make them easy to recognize. The present analytical results show that the most robust delimitation of *Cribrariaceae* is supported by only one synapomorphy: the presence of the plasmodic granules (character 52:1, FIG. 3). Although *Cribraria* has been described with a peridial net, *C. fragilis* and *C. zonatispora* (both included in our analyses) lack this feature (Lado et al. 1999, Estrada-Torres et al. 2001). The two species resemble each other in producing spores with warts and smooth areas and sharing a xeric environment and succulenticolous habit (Estrada-Torres et al. 2001). These two distinctive species are unresolved at the base of the *Cribrariaceae* (FIG. 2). The clade of 14 species that excludes these two *Cribraria* species is supported by the presence of the peridial net (character 27:1, FIG. 3).

Our analyses were not intended to elucidate the relationships within the *Cribrariaceae*, although the tree topology did reveal other groups, including one diagnosed by pulvinate nodes (character 31:1, FIG. 3) formed by *C. microcarpa*, *C. atrofusca*, *C. aurantiaca*, *C. tenella*, and *C. piriformis*. Another group comprising *C. rufa*, *C. vulgaris*, and *C. oregana* is supported by the synapomorphy of warty-reticulate spore ornamentation (character 45:1, FIG. 3). Nevertheless relationships among these species are undefined, and Jackknife and Bremer values do not support these two clades (FIG. 2).

Our analytical results support *Dictydium* within *Cribraria* (FIG. 2). Martin & Alexopoulos (1969), Farr (1976), Martin et al. (1983), and Keller & Braun (1999) have considered *Dictydium* a separate genus within the *Cribrariaceae*, but as species with intermediate features between *Cribraria* and *Dictydium* make differentiation difficult (Nannenga-Bremekamp 1962), there is no reason to keep these genera separate.

According to Martin & Alexopoulos (1969), *Dictydium*, which comprises three species (*Dictydium cancellatum* = *Cribraria cancellata*, *D. mirabile* = *C. mirabilis*, *D. rutilum* = *C. rutila*), is characterized by a peridial net composed of almost parallel ribs connected by thin transverse filaments and lacking expanded nodes or thickenings. However, the upper third of the peridial net in *Dictydium mirabile* (= *Cribraria mirabilis*) nearly lacks subparallel ribs, as is

characteristic in *Cribraria*. On the other hand, species placed in *Cribraria* (e.g., *C. splendens*) exhibit a peridial net having almost parallel ribs in the lower part of the sporotheca. Our taxonomic sampling of *D. cancellatum* and *D. mirabile* show both in a clade sister to *C. splendens* (FIG. 2). The Jackknife support is relatively low (64%) and the presence of ribs as a peridial net remains the only synapomorphy for this clade (character 29:1, FIG. 3).

Another significant taxonomic analytical result shows *Lindbladia tubulina*, currently classified in the *Cribrariaceae* sensu Martin et al. (1983) based on presence of plasmodic granules, grouping not in the *Cribrariaceae* (Clade C, FIG. 2) but in the *Reticulariaceae* (Clade R, FIG. 2). In observing that plasmodic granules are usually conspicuous in *Cribrariaceae*, Martin & Alexopoulos (1969) noted that in *Lindbladia* they are few and concolorous with membranes. In contrast, Hatano et al. (1996) observed in a more detailed analysis, "Most *Lindbladia* specimens when viewed by light microscopy showed darkly pigmented and conspicuous dictydine granules on the peridium." The *Lindbladia* specimens we studied have conspicuous plasmodic granules, corresponding more to Hatano et al. (1996) than Martin & Alexopoulos (1969).

Martin (1949) separated *Lindbladia* within the *Cribrariaceae* based primarily on the aethalioid and pseudoaethalioid habit and the lack of a peridial net. Hatano et al. (1996), however, mentioned the similarity of *Lindbladia* to *Dictydiaethalium*, *Enteridium*, and *Tubulifera* (= *Tubifera*) in the *Reticulariaceae* in its aethalioid and pseudoaethalioid habit. Our analytical results support the inclusion of *Lindbladia tubulina* in the *Reticulariaceae* by the sharing of a pseudoaethalium (FIG. 3). One reason for the contradictory literature descriptions (especially regarding the habit type) might be that gregarious forms of *Cribraria argillacea* have been confused with *Lindbladia* (Hatano et al. 1996). Our results place *Lindbladia tubulina* as sister of *Tubulifera arachnoidea* due to its spongy hypothallus (character 5:2, FIG. 3).

Our results show *Calonema foliicola* (*Trichiales*) as sister of the *Cribrariaceae*, although without Jackknife support (FIG. 2). This is relevant since relationships between *Cribrariaceae* and *Trichiales* have been inferred from cladistic analyses of molecular characters (elongation factor 1- α ; see Fiore-Donno et al. 2005). They concluded that *Trichiales* (represented by *Trichia persimilis* and *Arcyria denudata*) was the sister group of *Cribrariaceae* (represented by *Cribraria cancellata*). Although Fiore-Donno et al. (2005) included only three taxa in their analyses, their results agree with relationships obtained here based on morphological analyses of 4 exemplars of *Trichiales* and 16 of *Cribrariaceae*.

Relationship between *Cribrariaceae* and *Trichiales* (as represented by *Calonema* and *Metatrachia*, Clade 3) is supported by the synapomorphy of the calyculus and the warted spore ornamentation (character 24:1; character 44:1, FIG. 3). Hatano (1985) divided the spore ornamentation into several subtypes.

He mentioned that both *Cribrariaceae* and *Trichiaceae* share the ornamentation with warts, as seen by SEM. In the case of the *Cribrariaceae* warts are generally connected to each other in a row and form ridges.

Spore color and phylogeny

Lister (1925) classified the *Myxogastria* (*Myxomycetes*) in two major groups according to the color of spore mass: *Lamprospora*les with variously colored spores (seen in *Liceales* and *Trichiales* sensu Martin et al. 1983) but never violet-brown or purplish-gray and *Amaurospora*les with violet-brown to purplish-gray spores (seen in *Physarales* and *Stemonitales* sensu Martin et al. 1983) or colorless spores (seen in *Echinostelium*). In this context, any phylogenetic study has been driven to define these relationships. Nevertheless, the Fiore-Donno et al. (2005) molecular analyses support Lister's classification scheme, including *Stemonitales* and *Physarales* within a "dark-spored" clade, *Liceales* and *Trichiales* within a "clear-spored" clade, and *Echinosteliales* as sister of the "dark-" + "clear-spored" clade.

We coded several states for the whole range of spore color visible under light microscopy (i.e., colorless, yellow, brown-yellow, dark-brown, purple, red; see APPENDIX 2). Here the clades found by Fiore-Donno et al. (2005) differed from those we recovered from the EW and IW multi-state spore color analyses (FIGS. 1 and 2). Although the colorless-spored *Liceales* and *Trichiales* are related within Clade 2 and 3 in our results, these two orders do not form a monophyletic group. Our IW analysis includes all dark-spored taxa (*Physarales* and *Stemonitales* sensu Martin et al.) and the colorless-spored *Echinosteliales* (Lister 1925) within Clade 1 (FIG. 4).

In Fiore-Donno et al. (2005), the dark-spored representatives are not sister groups. Instead, the colorless-spored *Echinosteliales* are sister to the dark-spored *Stemonitales*, suggesting that dark spores appear in two separate clades with colorless spores appearing in several independent branches (FIG. 4).

Our results may appear to be biased in view of the different coding of spore color character as interpreted by Lister (1925, dark vs. clear spores). An additional analysis was performed to test the effect of including the spore color in three states as Lister (1925) proposed in his classification scheme. This character coding was as follows: dark spores (*Physarales* and *Stemonitales* sensu Martin et al.), clear spores (*Trichiales* and *Liceales* sensu Martin et al.), and hyaline spores (*Echinosteliales* sensu Martin et al.). The strict consensus tree shows a different topology with respect to the multi-state spore color code.

Results show the dark-spored representatives (Fiore-Donno et al. 2005) as a monophyletic group but including the colorless-spored *Echinosteliales*. Clear-spored orders (Fiore-Donno et al. 2005) are not a monophyletic group. Part of the *Liceales* (*Cribrariaceae*) and *Trichiales* is sister to the dark-spored clade, but

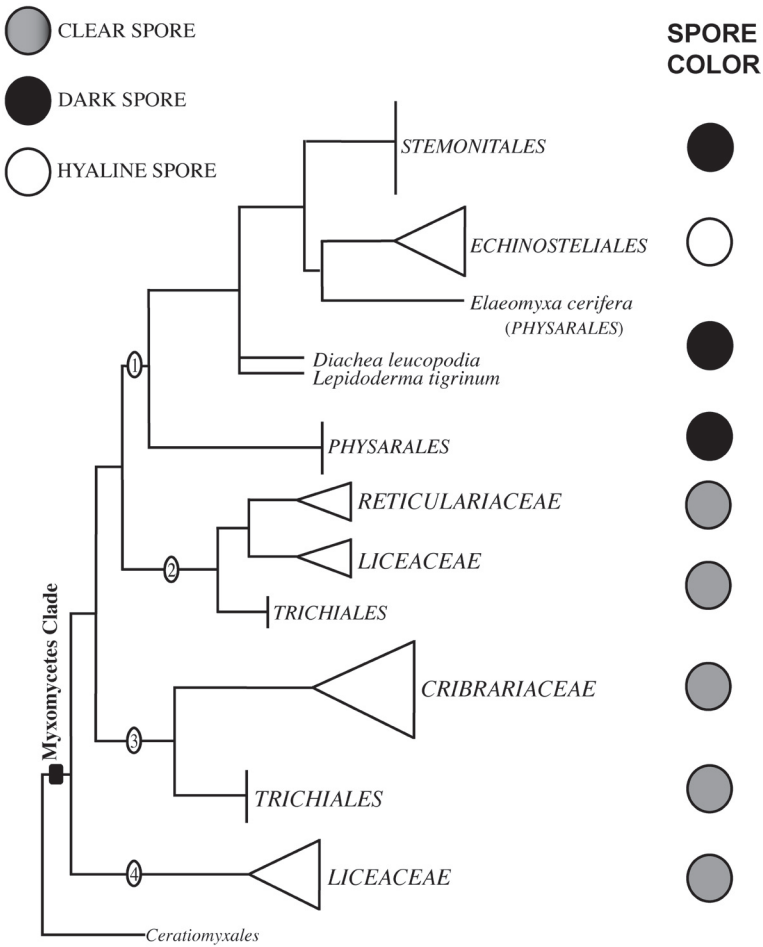


FIGURE 4. One of the MPTs based on implied weighting with the spore color (dark spores vs. clear spores) optimization as proposed by Fiore-Donno et al. (2005). This cladogram suggests that dark spores cluster in two separate clades, whereas clear-spores appeared in several branches independently.

another clade with the *Reticulariaceae* and part of the *Liceaceae* is sister to the dark-spored + *Cribrariaceae* and *Trichiales* clades.

Different spore color character coding (three state vs. multi-state) produces different hypotheses of internal relationships among the myxomycete orders. Fiore-Donno et al. (2005) observed that spore color might be a consistent morphological marker for slime mold phylogeny, but our incongruent trees

contradict their assumptions. We choose the multi-state code in order to include the whole variation visible under microscope.

Multi-state coding of spore color, together with the other morphological characters analyzed, were important in defining the dark-spored clade groups (*Stemonitales* and *Physarales*), therefore the multi-state coding is better than the two state code to resolve the phylogenetic history of *Myxomycetes*.

Kalyanasundaram et al. (1994), who studied the spore wall pigments of several *Myxomycetes*, found that melanin is the only pigment present in the dark-spored orders *Physarales* and *Stemonitales*. They also found melanin in the clear-spored *Liceales* and *Trichiales* and explained the clear spore color by suggesting that other pigments were present that masked the melanin. Further evaluation of additional spore color pigments is needed.

In conclusion, our phylogenetic analytical results derived from morphological characters indicate that the *Liceales* is not a monophyletic group, as several authors have proposed (Alexopoulos 1976, Eliasson 1977, Eliasson et al. 1991). The analyses also suggest that *Dictydium* should be considered part of *Cribraria* (as proposed by Nannenga-Bremekamp, 1962) and not a separate genus, given that a peridial net, the presence of ribs, and plasmodic granules are shared in both genera. *Cribraria* should be amended to include species lacking a peridial net in the sporophores, such as *C. zonatispora* (Lado et al. 1999) and *C. fragilis* (Estrada-Torres et al. 2001). Although *Lindbladia tubulina* is now included in the *Reticulariaceae*, analysis of more characters or the inclusion of *Cribraria cribrarioides* (= *Lindbladia cribrarioides*) might help to clarify their phylogenetic position, either in the *Cribrariaceae* or the *Reticulariaceae*, as our results suggest. Our study also reveals the phylogenetic value of morphological characters. Clark (2000) suggested that myxomycete morphological analysis is problematic due to character plasticity and the difficulty in growing fruitbodies in the laboratory. Nevertheless, careful examination of morphological variation, unbiased character assessment, and adequate character state coding methodology will help reveal patterns of phylogenetic congruence among characters, which could be useful to define the *Cribrariaceae* in a cladistic framework.

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Appendix 2. Characters and character states (Morphology of the sporophore)

- 1.- AETHALIUM: A relatively large, sessile, round or mound-shaped fruiting body formed from all or a major portion of plasmodium. Common in some members of *Liceales* and *Physarales*. 0 = absence; 1 = presence.
- 2.- PLASMODIOCARP: A sessile, branched, ring-shaped, or netted type of fruiting body formed when a plasmodium becomes concentrated in its main veins during fruiting. 0 = absence; 1 = presence.
- 3.- SPOROCARP: A type of fruiting body formed when a plasmodium breaks up into a small portions, each of which develops into a single stalked or sessile unit. It is the most common fruiting body in taxa of all orders in *Myxomycetes*. 0 = absence; 1 = presence.
- 4.- PSEUDOAEATHALIUM: A type of fruiting body that consist of a mass of sporangia tightly packed together to resemble an aethalium. It is present in *Dictydiaethalium plumbeum*, *Tubulifera arachnoidea* (both members of *Reticulariaceae*); *Lindbladia tubulina* (*Cribrariaceae*), and *Metatrachia vesparia* (*Trichiaceae*). 0 = absence; 1 = presence.
- 5.- TYPE OF HYPOTHALLUS: The hypothallus is a layer deposited by the plasmodium at the time of fruiting, located at the base of sporangia in the substrate. Normally, in mostly of *Myxomycetes*, the hypothallus may appear as a delicate or tough membrane, but it may appear conspicuous and spongy or limy, like occur in *Lindbladia tubulina*, *Tubifera microsperma* or *Mucilago crustacea*. It could be also mucilaginous, massive and solid as in *Ceratiomyxales*. 0 = massive solid; 1 = membranous; 2 = spongy.
- 6.- STALK: The stalk is a structure which support the sporotheca (the structure where the spores develop), raising up from the substrate. It may display a diverse range of length, thickness, colors and textures (Farr 1981). 0 = absence; 1 = presence.
- 7.- NATURE CONTENT OF STALK: The stalk may be hollow, as it happens in most of the *Stemonitales*, or filled with several kinds of materials as occur in *Liceales* or *Trichiales*. 0 = solid; 1 = hollow.
- 8.- CYSTS IN THE STALK: The stalk of some species of *Trichiales* are filled with vesicular structures resembling spores, but larger, which are called cysts. In our data set, the species that have cysts as filling material are *Hemitrichia calyculata*, *Calonema foliolica*, and *Trichia decipiens*. 0 = absence; 1 = presence.
- 9.- LIME IN THE STALK: Another filling material frequently present in the stalk is calcium carbonate granules. It is present in some species of *Physarales*, such as *Diachea leucopodia*, and *Lepidoderma tigrinum*. 0 = absence; 1 = presence.
- 10.- FIBERS IN THE STALK: According with Gray and Alexopoulos (1968), the stalk in the species of *Comatracha* and *Clastoderma* consists of a system of parallel fibers. In our matrix, this character is present in *Enerthenema papillatum*, *Lamproderma scintillans*, and *Comatracha laxa*. 0 = absence; 1 = presence.
- 11.- CAPILLITIUM: A system of sterile threads or tubules found within the spore mass of the fruiting body of many *Myxomycetes*. It may aid to the dissemination of spores. With the exception of the *Liceales*, it is present in most of the other species of *Myxomycetes*. The capillitium is a very important and useful character in the identification of orders, families, genera and species (Farr 1981). 0 = absence; 1 = presence.
- 12.- TYPE OF CAPILLITIUM: The capillitium may be formed by threads, i.e. solid structures, as in *Calomyxa metallica*, or tubules i.e. hollow structures, as in the most of *Physarales*, *Trichiales* and *Stemonitales*. 0 = hollow; 1 = solid.
- 13.- CAPILLITIUM ORNAMENTATION: It is applicable for those capillitial threads or tubules not smooth, which have the surface marked or sculptured with spines, warts, dots, rings, reticules, etc. It is an essential character in the generic and specific distinction of *Trichiales*. 0 = absence; 1 = presence.

- 14.- CAPILLITIUM COLOR: This refers to the color of the capillitial threads or tubules observed under the light microscope. Generally in the *Stemonitales* the capillitium is dark and slender, while in the *Trichiales* it is mostly pale or brightly colored. Some species of *Physarales* have a pale capillitium. We coded according with the range-color we observed in the taxa we studied. 0 = pale; 1 = reddish; 2 = yellow; 3 = dark brown.
- 15.- ATTACHMENT OF THE CAPILLITIUM: The capillitium threads or tubules may be free or attached either to the columella or the peridium. 0 = free; 1 = attached.
- 16.- CAPILLITIUM ATTACHMENT TO THE COLUMELLA: It is mostly present in the *Stemonitales* and *Echinosteliales*, but some members of *Physarales* (*Diachea leucopodia* and *Elaeomyxa cerifera*) have it too. 0 = absence; 1 = presence.
- 17.- CAPILLITIUM ATTACHMENT TO THE PERIDIUM: In some species of *Myxomycetes*, the tips of the elements of the capillitium stay attached to the peridium, leaving fragments of it in the surface of the sporotheca. In some *Trichiales* the capillitium is adhered to the calyculus, the persistent like-cup peridium in the base of their sporothecae. 0 = absence; 1 = presence.
- 18.- PLACE OF ATTACHMENT BETWEEN CAPILLITIUM AND COLUMELLA: The capillitium may arise along all the columella or only at the apex of this structure. 0 = to the apex; 1 = along the columella.
- 19.- BRANCHING OF THE CAPILLITIUM: The elements of the capillitium may be simple, branched or branched-anastomosed forming a complex reticulated structure. 0 = simple; 1 = branched; 2 = anastomosed.
- 20.- CAPILLITIUM SUPERFICIAL NET: Some species of *Myxomycetes* have a superficial net formed by the anastomosis of the capillitial elements in the surface of the sporotheca, as present in species of the genus *Stemonitis*. 0 = absence; 1 = presence.
- 21.- CALCIUM CARBONATE IN THE CAPILLITIUM: In the *Physarales*, the capillitium may be entirely limy, such as in a typical *Badhamia*, or may consist of a system of hyaline tubules supporting calcareous nodes like in *Physarum*. In other families lime is rarely present in the capillitium even though lime may be characteristically deposited in other parts of the fructification. 0 = absence; 1 = presence.
- 22.- BIREFRINGENCE OF CAPILLITIUM: Birefringence is found only in the capillitium of some *Trichiales*. Most of the species that have spirals on the capillitium show very brilliant birefringence (Nannenga-Bremekamp 1982). We used this character in order to define the relationships within the *Trichiales* order. 0 = absence; 1 = presence.
- 23.- PERIDIUM: The peridium (plural: peridia) is a covering that encloses the spore mass (plus other structures) of a fruiting body. The peridium may be tough, thin or delicate, persisting in the mature reproductive body or disappearing, partially or totally, after the spores become mature. 0 = persistent; 1 = partially persistent; 2 = fugacious.
- 24.- CALYCLUS: The calyculus is the persistent basal portion of the peridium, forming a cup-shaped structure at the bottom of the sporothecae in some species of *Hemitrichia*, *Arcyria* or *Trichia* in the order *Trichiales*, and *Cribraria* or *Lindbladia* in the order *Liceales*. 0 = absence; 1 = presence.
- 25.- DEPTH OF THE CALYCLUS: The calyculus may be shallow, ranging 1/3 or less than the height of the sporotheca, or deep, occupying more than 1/3. 0 = shallow; 1 = deep.
- 26.- MARGIN OF THE CALYCLUS: The calyculus may have smooth margin or have projections forming an irregular margin with teeth or ribs. 0 = irregular; 1 = regular.
- 27.- PERIDIAL NET: This is a persistent peridium which remains as a reticulate structure in the sporotheca of some members of the *Cribrariaceae* (Martin and Alexopoulos 1969). 0 = absence, 1 = presence.
- 28.- THREADS FREE IN THE PERIDIAL NET: The free ending threads in the peridial nets of certain species of *Cribraria*. 0 = absence; 1 = presence.

- 29.- RIBS IN THE PERIDIAL NET: Sub parallel elements of the peridial net, always inter connected by short transverse threads. They may arise from the base of the sporotheca or from the calyculus. 0 = absence; 1 = presence.
- 30.- NODES IN THE PERIDIAL NET: An expanded junction of the threads in the peridial net of the reproductive body of some members of *Cribrariaceae*. 0 = absence; 1 = presence.
- 31.- TYPE OF NODES: The nodes can be flattened or thickened by the aggregation of the plasmodic granules. Thickened nodes are pulvinate in lateral view. 0 = not pulvinate; 1 = pulvinate.
- 32.- PERIDIAL COLLAR: The remains of the peridium around the stalk at the base of the sporotheca is called a peridial collar. 0 = absence; 1 = presence.
- 33.- PERIDIAL PLATES: The peridium of some species of *Licea* is composed by polygonal plates which give a polyhedral shape to the sporotheca. 0 = absence; 1 = presence.
- 34.- LIME IN THE PERIDIUM: CaCO_3 may be present in the peridium of many species of *Physarales*, either as crystals or granules. 0 = absence; 1 = granules; 2 = crystals.
- 35.- COLUMELLA: A sterile structure that extends into the spore mass from below, as an extension of the stalk. It occurs in certain genera belonging to the orders *Echinosteliales*, *Physarales*, and *Stemonitales*. 0 = absence; 1 = presence.
- 36.- TYPE OF COLUMELLA: The columella may be spherical, hemispherical club-shaped, elongated, dome-shaped or reduced as a thickened sporangial base. In our study, only elongate and hemispherical states were present in the taxa included. 0 = elongate; 1 = hemispherical.
- 37.- LIME IN COLUMELLA: As occurs with other structures, CaCO_3 may be present in the form of granules in the columella. 0 = absence; 1 = presence.
- 38.- COLUMELLA EXTENDING: The length of the columella can reach the apex, the middle portion, or only at the base of the sporotheca. 0 = to the apex of sporotheca; 1 = to the middle of sporotheca; 2 = to the base of sporotheca.
- 39.- PROSPORES: An uninucleate segment of the protoplasm. It is found on the surface of the columns of the reproductive fructification of the *Ceratiomyxales*, and gives rise to one sporocarp. 0 = absence; 1 = presence.
- 40.- TYPE OF MYXOSPORES: It refers to the shape of the spores. It is an important character in *Myxomycetes* taxonomy. In this work we don't include all existing forms, but only the states for the taxa we analyzed. 0 = globose; 1 = elliptical; 2 = angular; 3 = sub-globose; 4 = discoid.
- 41.- COLOR OF MYXOSPORES: A further important feature is spore color, which appears darker in the mass in reflected light under the hand lens or dissecting microscope than with transmitted light as seen under the microscope. We coded this character with observations under the light microscope in order to define more precisely the range of color states present in all *Myxomycetes*. 0 = hyalines; 1 = reddish; 2 = yellowish; 3 = brown-dark; 4 = purple; 5 = brown-yellowish.
- 42.- NUMBER OF SPORES FORMED IN THE SPOROTHECA: The sporotheca of some protostelids form a unique spore in their fruit body, such as *Ceratiomyxa* (outgroup). The *Myxomycetes* generally produce many spores by sporotheca. 0 = monosporic; 1 = multisporic.
- 43.- ORNAMENTATION OF THE MYXOSPORES: The surface of the spore, as seen under light microscope, might be smooth or with projections of diverse forms. 0 = absence; 1 = presence.
- 44.- ORNAMENTATION WITH WARTS: The warts are short projections, dispersed in surface of the spore and obtuse in the apex. 0 = absence; 1 = presence.
- 45.- ORNAMENTATION WARTED-RETICULATED: The warts might be lined, with the lines forming a reticulum. It is present in this study only for three species of the *Cribrariaceae* (*C. rufa*, *C. oregana* and *C. vulgaris*). 0 = absence; 1 = presence.
- 46.- ORNAMENTATION WITH BANDED-RETICULATED: This ornamentation is formed by bands that form a reticulate. Representatives of the *Liceales* and *Trichiales* share this type of ornamentation. 0 = absence; 1 = presence.
- 47.- ORNAMENTATION WITH RUGULOSE: It is formed by folds in the surface of the spore. 0 = absence; 1 = presence.

- 48.- ORNAMENTATION WITH SPINES: This ornamentation is formed by conical with sharp apex. 0 = absence; 1 = presence.
- 49.- ORNAMENTATION WITH DOTS: Very tiny projections giving a dotted appearance on the surface of the spore. 0 = absence; 1 = presence.
- 50.- ORNAMENTATION WITH CRESTAE: This ornamentation is formed by projections giving a wavy aspect. 0 = absence; 1 = presence.
- 51.- MYXOSPORE WALL: The myxospore wall might be homogenous in thickness or have a thinner and paler area at one pole, as seen in light microscopy. 0 = homogenous; 1= with a diffuse thinner wall at one pole.
- 52.- PLASMODIC GRANULES: Microscopic, usually dark-colored structures found in the fruiting bodies of *Cribrariaceae*. They are called also "dictydine granules" (Martin and Alexopoulos 1969) or "lime globules" (Nannenga-Bremekamp 1991). 0 = absence; 1 = presence.
- 53.- PSEUDOCAPILLITIUM: A system of irregular plates, tubes, or threadlike elements occurring within the spore mass, and suggestive of a true capillitium but not formed in the same way; it is characteristic of some members of the *Liceales*. 0 = absence; 1 = presence.
- 54.- THREAD PHASE: It is a tetra nucleate and elongate thread phase produced immediately after the germination of the spores of the *Ceratiomyxales*. 0 = absence; 1 = presence.